AN *IN VIVO* AND *IN VITRO* MODEL FOR CUTANEOUS PHOTOAGING AND OXIDATIVE DAMAGE

Background of the Invention

Chronic sun exposure eventuates in wrinkling, sagging, pigmentary alterations, and skin cancers which are characteristic of sun-damaged skin, and collectively referred to as photoaging (Kligman, A. M., *JAMA*, 1969, 210:2377-2380; Gilchrest, B.A., *J. Am. Acad. Dermatol.*, 1989, 21:610-613,). The major histopathologic alteration of photoaged skin is the accumulation of material which, on routine histopathologic examination, has the staining characteristics of elastin and is, thus, termed solar elastosis. Immunohistochemical staining has shown the poorly-formed fibers comprising solar elastosis to be composed of elastin (Chen et al., *J. Invest. Dermatol.*, 1986, 87:334-337; Mera et al., *Br. J. Dermatol.*, 1987, 117:21-27) fibrillin (Chen et al., *J. Invest. Dermatol.*, 1986, 87:334-337; Dahlback et al., *J. Invest. Dermatol.*, 1990, 94:284-291,; Bernstein et al., *J. Invest. Dermatol.*, 1994, 103:182-186) and versican, the normal components of elastic fibers (Zimmerman et al., *J. Cell. Biol.*, 1994, 124:817-825). A coordinate increase in elastin, fibrillin and versican mRNAs has been demonstrated in fibroblasts derived from photodamaged skin, as compared to fibroblasts derived from normal skin from the same individuals (Bernstein et al., *J. Invest. Dermatol.*, 1994, 103:182-186). Elevated elastin mRNA levels in sun-damaged skin result from enhanced elastin promoter activity, as shown by transient transfections of fibroblasts with a DNA construct composed of the human elastin promoter linked to the chloramphenicol acetyltransferase (CAT) reporter gene (Bernstein et al., *J. Invest. Dermatol.*, 1994, 103:182-186).

A transgenic mouse line expressing the 5.2 kb human elastin promoter linked to a chloramphenicol acetyltransferase reporter gene (CAT) has been developed which models cutaneous

photoaging (Bernstein et al., *J. Invest. Dermatol.*, 1995, 105, 269-273). Although phenotypically normal, the cells in these mice possess the human elastin promoter/CAT construct, allowing elastin promoter activity to be measured in response to stimuli such as ultraviolet radiation (UV). Four or five day old mice which have not yet developed hair, and fibroblast cultures derived from their skin, have been demonstrated to provide a rapid and sensitive means of identifying compounds capable of inhibiting cutaneous photodamage (Bernstein et al., *J. Invest. Dermatol.*, 1995, 105, 269-273; Bernstein et al., *Photochem. Photobiol.*, 1996, 64:369-74; Bernstein et al., *J. Am. Acad. Dermatol.*, 1997, 37:725-729).

UV from the sun also damages skin by the generation of reactive oxygen species (Miyachi, Y. J., *Dermatol. Sci.*, 1995, 9:79-86). Reactive oxygen species may form immediately as a result of UV exposure, or result from the inflammatory response which often follows UV-induced injury. Although the erythema of a sunburn is clinical evidence of damage from UV, an inflammatory infiltrate may be evident histopathologically even in the absence of erythema, and may result in continued exposure of the dermis to free radicals, days after the UV-induced damage has occurred (Kligman, A. M., *JAMA*, 1969, 210:2377-2380; Lavker et al., *J. Am. Acad. Dermatol.*, 1995, 32:53-62). The role of free radicals in cutaneous photodamage has been well documented (Ranadive, N.S. and Menon, I.A., *Pathol. Immunopathol. Res.*, 1986, 5:118-139; Miyachi, Y and Imamura, S., *Photodermatol. Photoimmunol. Photomed.*, 1990, 7:49-50; Miyachi, Y. J., *Dermatol. Sci.*, 1995, 9:79-86; and Peak et al., *Photochem. Photobiol.*, 1991, 54:197-203). UV-induced free radical generation in skin has been demonstrated (Peak et al., *Photochem. Photobiol.*, 1991, 54:197-203; and Norins, A.L., *J. Invest. Dermatol.*, 1962, 39:445-448). In addition, some enzymes which protect against oxidative damage, such as superoxide dismutase and catalase, are depleted after

UV exposure (Pence, B.C. and Naylor, M.F., *J. Invest. Dermatol.*, 1990 95:213-216; Maeda et al., *Photochem. Photobiol.*, 1991, 54:737-740; Shindo, Y and Hashimoto, T., *J. Dermatol. Sci.*, 1997, 14:225-232), and antioxidants that scavenge free radicals have demonstrated protection against UV (DeRios et al., *J. Invest. Dermatol.*, 1975, 70:123-125; and Bissett et al., *J. Soc. Cosmet. Chem., 1992,* 43:85-92). Investigators have recently demonstrated elastin mRNA production in response to free radicals generated using a xanthine and xanthine oxidase system *in vitro*, providing evidence for the role of oxidative stress in the generation of solar elastosis (Kawaguchi et al., *Free Radical Biol. Med.*, 1997, 23:162-165).

A transgenic hairless mouse model has now been developed which expresses a full length or truncated human elastin promoter/reporter gene. These transgenic hairless mice express human elastin promoter activity in a tissue-specific and developmentally regulated manner. Not only can quantitative data be obtained in these mice or fibroblasts derived therefrom after only a single exposure to ultraviolet radiation, but these hairless mice can also be used in long term phototoxicity studies. A single exposure to UVB in transgenic hairless mice having a truncated elastin promoter resulted in about a 20 to 30 fold increase in elastin activity as compared to an 8-fold increase reported for prior art models expressing the full length promoter. Accordingly, this transgenic hairless mouse and fibroblasts derived from this hairless mouse are useful as *in vivo* and *in vitro* models to study cutaneous photoaging and in the identification of agents which may protect against photodamage.

Further, reactive oxygen species are also believed to stimulate elastin production at the promoter level in fibroblasts derived from these mice. Accordingly, the present invention also relates to an *in vitro* system and method for

identifying agents capable of protecting against oxidative damage via a mouse fibroblast culture derived from a transgenic hairless mouse capable of expressing a full length or truncated human elastin promoter and a means for generating reactive oxygen species within the mouse fibroblast cultures.

Summary of the Invention

An object of the present invention is to provide a transgenic hairless mouse capable of expressing a full length or truncated human elastin promoter.

Another object of the present invention is to provide mouse fibroblast cultures derived from a transgenic hairless mouse capable of expressing a full length or truncated human elastin promoter.

Another object of the present invention is to provide methods of identifying compounds capable of inhibiting cutaneous photodamage using either the transgenic hairless mouse or fibroblasts derived from these mice.

Another object of the present invention is to provide an *in vitro* system for identifying agents capable of inhibiting or preventing oxidative damage comprising a mouse fibroblast culture derived from a transgenic hairless mouse capable of expressing a full length or truncated human elastin promoter and a means for generating reactive oxygen species within the mouse fibroblast culture.

Yet another object of the present invention is to provide a method of identifying agents capable of inhibiting or preventing oxidative damage using this *in vitro* system.

Detailed Description of the Invention

In the present invention, a transgenic hairless mouse model has been developed which permits the investigation of human elastin promoter activity in response to ultraviolet irradiation both *in vivo* by direct irradiation of mouse skin, and *in vitro* by irradiation of dermal fibroblasts grown from

skin explants of these mice. In a preferred embodiment the hairless mouse used in the production of these transgenic mice is of the strain Crl:SKH1-hrBR (Charles River) as this hairless strain of mice is well characterized and used routinely in preclinical dermatological and photobiological research. The transgenic hairless mice of the present invention are capable of expressing a full length or truncated elastin promoter. By "truncated human elastin promoter" it is meant a human elastin promoter shorter than the full length 5.2 kb human elastin promoter such as pEP62, pEP35, pEP10, pEP27, and pEP6 (Kahari et al., *J. Biol. Chem.*, 1990, 265(16):9485-9490) which is activated by UV. In a preferred embodiment, the truncated elastin promoter is pEP6. It is also preferred that the promoter be linked to a reporter gene such as the chloramphenicol acetyltransferase reporter gene (CAT) for ease in detecting activity of the full length or truncated promoter.

Other *in vivo* models of photoaging require numerous treatments over long periods of time to demonstrate a measurable effect. For example, experimentally produced elastosis in mice was first produced by Sams et al. using very large amounts of ultraviolet radiation (*J. Invest. Dermatol.*, 1964, 43:467-471). In these studies, one group of mice received 1,040 human minimal erythema doses (MEDs) over 3 months from a bank of fluorescent tubes, while another group received 13,000 MEDs given over 52 weeks in 260 treatments. Elastosis was demonstrated by histochemical staining for elastin and, in irradiated mice, demonstrated an increased elastin staining. Since this initial report, a number of researchers have used murine models of cutaneous photoaging evaluating the production of dermal elastosis (Sams et al., *J. Invest. Dermatol.*, 1964, 43:467-471; Nakamura, K. and Johnson, W.C., *J. Invest. Dermatol.*, 1968, 51:253-258; Berger et al., *Arch. Dermatol. Res.*, 1980, 269:39-49; Kligman, L.H., *Arch. Dermatol. Res.*, 1982, 272:229-238; Kligman et al., *J.*

Invest. Dermatol., 1982, 78:181-189; Poulsen et al., *Br. J. Dermatol.*, 1984, 110:531-538; Kligman et al., *J. Invest. Dermatol.*, 1985, 84:272-276; Bissett et al., *Photochem. Photobiol.*, 1987,, 1987 46:367-376; Bissett et al., *Photochem. Photobiol.*, 1989, 50:763-769; Wulf et al., *Photodermatology* 6:44-51, 1989; Kligman, L.H. and Sayre, R.M., *Photochem. Photobiol.*, 1991, 53:237-242; and Moran, M. and Granstein, R.D., *J. Invest. Dermatol.*, 1994, 103:797-800). The number of treatments with ultraviolet radiation in these studies ranges from 36 to 260 given over 13 to 62 weeks.

A homozygous line of transgenic mice expressing the 5.2-kb human elastin promoter linked to a CAT reporter gene has been disclosed (Hsu-Wong et al., *J. Biol. Chem.*, 1994, 269:18072-18075). U.S. Patent 5,648,061 discloses the use of this transgenic mouse model to investigate human elastin promoter activity in response to ultraviolet irradiation both *in vivo* by direct irradiation of mouse skin, and *in vitro* by irradiation of dermal fibroblasts grown from skin explants. These mice express the human elastin promoter in a tissue-specific and developmentally regulated manner. Further, a dose-response relationship for elastin promoter activity after only a single dose of UV has been observed. In these mice a single dose of UVB (491.4 mJ/cm^2) resulted in up to an 8.5-fold increase in promoter activity, while a more modest 1.8-fold increase was measured with UVA (38.2 J/cm^2). However, mice four or five days old must be used since at this age, visible hair growth is not yet present.

Further, experiments with UVA treatment in these mice, and more particularly fibroblasts derived from these mice, require addition of 8-methoxypsoralen prior to UVA exposure to achieve a significant increase in elastin promoter activity. The combination of 8-methoxypsoralen (8-MOP) and UVA is referred to routinely in the art as PUVA. Treatment of skin diseases with PUVA results in clinical alterations in

treated skin similar to those observed in chronically photodamaged skin. PUVA-treated patients develop non-melanoma skin cancers, pigmentary alterations and wrinkling characteristics of sun-induced changes. Fibroblast cultures derived from the mice of Hsu-Wong et al. treated with 8-MOP or UVA alone exhibited no significant change in CAT activity as compared to untreated controls. However, PUVA-treated cell cultures demonstrated 2.6-, 13.2- and 2.0-fold increases in CAT activity in response to 1 J/cm^2 of UVA with 8-MOP doses of 0.3, 1.0, and 3.0 μg/ml of 8-MOP, respectively.

In the hairless mice of the present invention, not only is a quantifiable increase in elastin promoter activity in UVB-treated mice observed after a single dose of UVB, but these mice can also be used in long term phototoxicity studies. Further, a 20 to 30 fold increase in promoter activity was observed in mice of the present invention expressing a truncated human elastin promoter following a single dose of UVB, thus demonstrating that transgenic hairless mice capable of expressing a truncated elastin promoter provide a more sensitive model as compared to prior art mice expressing the full length promoter. With this increase in sensitivity to UV, it is believed that application of psoralen to fibroblasts and/or mice will no longer be required in experiments investigating UVA effects.

The present invention also relates to methods of identifying compounds capable of inhibiting cutaneous photodamage with this transgenic hairless mouse model. In one embodiment a test compound is applied to the skin of a transgenic hairless mouse capable of expressing a full length or truncated human elastin promoter. The transgenic hairless mouse is then exposed to ultraviolet radiation, either solar simulating, UVB or UVA, and human elastin promoter activity in the mouse is determined. The human elastin promoter activity is then compared to that in control transgenic hairless mice also exposed to an equivalent dose of

ultraviolet radiation which were not treated with the test compound to determine whether or not the test compound provided protection against the ultraviolet radiation. In another embodiment, fibroblast cells derived from a transgenic mouse capable of expressing a full length or truncated human elastin promoter are treated with a test compound. The treated fibroblast cells are then exposed to solar simulating, UVB or UVA radiation and human elastin promoter activity in the fibroblast cells is determined. This activity is compared to control fibroblast cells from the transgenic mice exposed to the same dose of solar simulating, UVB or UVA radiation but which were not treated with the test compound to determine if the test compound provided protection against the exposure.

Oxidative damage is also believed to play a role in dermal damage from UV radiation. Thus, the generation of photoaging, clinically evident as wrinkling and sagging of skin, may result more from free radical-induced mechanisms than UV-induced skin cancers which originate in the epidermis. Evidence for this includes the greater sensitivity of fibroblasts to free radical-induced damage as compared to keratinocytes (Applegate, L.A. and Frenk, E. *Photodermatol. Photoimmunol. Photomed.*, 1995, 11:95-101; Moysan et al., *Photodermatol. Photoimmunol. Photomed.*, 1995, 11:192-197; Masaki, H. and Sakurai, H. *J., Dermatol. Sci.*, 1997, 14:207-216), and the fact that circulating inflammatory cells which produce free radicals course through the dermis and less frequently invade the epidermis. Also the longer wavelengths of UV, which produce less direct DNA damage (Setlow, R.B., *Science*, 1966, 153:379-386) but may exert their deleterious effects mainly through oxidative mechanisms, penetrate more deeply into skin, depositing much of their energy in the dermis. Thus, free radical mechanisms of damage may be the primary means by which UVA-induced photoaging takes place.

A number of effective sunscreens for blocking UVB are currently on the market, and increasing amounts of UVA

protection are being incorporated into sunscreens to obtain higher sun protection factors. Further improvements are likely to result from incorporating effective free radical scavengers into currently available sunscreens. Accordingly, there is a need for a system of identifying agents which inhibit or prevent oxidative damage from the sun.

Generation of reactive oxygen species via a hypoxanthine and xanthine oxidase system in dermal fibroblasts derived from transgenic mice expressing a full length human elastin promoter results in a measurable increase in elastin promoter activity. Further, this increase can be eliminated by the addition of catalase, an enzyme known to protect against oxidative damage. Accordingly, incorporation of a means for generating reactive oxygen species such as a hypoxanthine and xanthine oxidase system within mouse fibroblast cultures derived from the transgenic hairless mice of the present invention is believed to provide a sensitive system for evaluating agents which may prevent oxidative damage. Using this system, agents which may protect against the oxidative damage resulting from UV exposure may be rapidly screened, and promising candidates identified for further study and eventual incorporation into sunscreens.

Test agents suspected of providing protection against oxidative damage can be added to the hairless mouse fibroblast culture prior to addition of the means for generation of reactive oxygen species. The means for generating reactive oxygen species is then added and human elastin promoter activity is determined in the mouse fibroblast culture after a selected time period. The time period for determination of human elastin promoter can be selected in accordance with routine experiments wherein optimum time span for incubation of fibroblasts, derived from the skin of the transgenic mice, with a hypoxanthine and xanthine oxidase system is determined. More specifically, optimum time span for incubation is determined by exposing cells to a hypoxanthine and xanthine

oxidase system for increasing amounts of time, and determining promoter activity at various times throughout a 24 hour incubation. Optimum time is determined as the point at which CAT activity peaks.

In a preferred embodiment, elastin promoter activity is determined by measuring expression of the reporter gene, i.e. the CAT activity, in the hairless mouse fibroblast culture. Human elastin promoter activity in the hairless mouse fibroblast culture exposed to the test agent is then compared to elastin promoter activity in a control hairless mouse fibroblast culture not exposed to the test agent but still exposed to the means for generating reactive oxygen species. Agents providing protection against oxidative damage are identified as those test agents which decrease human elastin promoter activity in the hairless mouse fibroblast culture exposed to the test agent and the means for generating reactive oxygen species as compared to the control hairless mouse fibroblast culture.

The following nonlimiting examples are provided to further illustrate the present invention.

Examples

Example 1: Transgenic mice expressing the human elastin promoter

Homozygous lines of hairless transgenic mice of the strain Crl:SKH1-hrBR (Charles River) expressing either the full length 5.2-kb human elastin promoter linked to a CAT reporter gene or the truncated human elastin promoter, pEP6 (Kahari et al., *J. Biol. Chem.*, 265(16):9485-9490, 1990), linked to a CAT reporter gene were produced in accordance with known methods for production of transgenic mice.

Example 2: Fibroblast Cultures Expressing the Human Elastin Promoter

Fibroblasts derived from the skin of homozygous lines of transgenic hairless mice expressing either the full length 5.2-kb human elastin promoter or the truncated elastin promoter pEP6 (Kahari et al., *J. Biol. Chem.*, 1990, 265(16):9485-9490), linked to a CAT reporter gene which enables measurement of elastin promoter activation, as previously described (Bernstein et al., *J Invest Dermatol*, 1995, 105, 269-273; and Bernstein et al., *Photochem Photobiol*, 1996, 64:369-74) are utilized. Although phenotypically normal, these mice express the human elastin promoter when assayed for CAT activity (Hsu-Wong et al., *J. Biol. Chem.*, 1994, 269: 18072-18075; Bernstein et al., *J Invest Dermatol*, 1995, 105, 269-273; and Bernstein et al., *Photochem Photobiol*, 1996, 64:369-74).

Fibroblast cultures are established from the skin of these hairless transgenic mice by explanting tissue specimens onto plastic tissue culture dishes and allowing cells to migrate to the area of the dish surrounding the explants. The primary cultures are maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine and antibiotics, at 37°C. After exposure to ultraviolet radiation, the cells are incubated in DME medium supplemented with 10% fetal calf serum for 24 hours, then harvested for determination of CAT activity as described in Example 4.

Example 3: Exposure of fibroblast cultures to free radicals generated by hypoxanthine/xanthine oxidase

DMEM with 10% FCS is removed, cells are rinsed in phosphate buffered saline (PBS), and DMEM is replaced without the addition of FCS. Both 500 μM hypoxanthine (Sigma Chemical Co., St. Louis, MO) and 80 mU/ml xanthine oxidase (Sigma

Chemical Co., St. Louis, MO) are then added to fibroblast cultures and incubated at 37°C for a selected time period. These concentrations of hypoxanthine and xanthine oxidase were selected based on previous work by Mitchell et al. (*Biochemistry* 29:2802-2807, 1990). The time for incubation of cells with hypoxanthine/xanthine oxidase can be selected by exposing cells to hypoxanthine/xanthine oxidase for 15, 30, 60, 90 and 120 minutes and determining CAT activity as outlined in Example 4.

After exposure to hypoxanthine/xanthine oxidase, cells are rinsed in PBS and incubated in DMEM with FCS for the time in which maximal promoter activation is determined to occur after exposure to hypoxanthine/xanthine oxidase. Control cells are treated in an identical fashion without the addition of hypoxanthine/xanthine oxidase.

In addition to hypoxanthine/xanthine oxidase treatment alone, cells are also treated with hypoxanthine/xanthine oxidase plus 10,000 U/ml of catalase (Sigma Chemical Co., St. Louis, MO) co-incubated for a selected time, and harvested as outlined above. Fibroblasts from the hairless mice representing the same litter are used for any given experiment. Four dishes of cells are used for each experimental condition (control, hypoxanthine/xanthine oxidase, and hypoxanthine/xanthine oxidase+catalase), and experiments are repeated in duplicate, yielding a total of eight values for each experimental condition.

The effect of hypoxanthine/xanthine oxidase and hypoxanthine/xanthine oxidase+catalase on cell viability is determined using the trypan blue (Sigma Chemical Co., St. Louis, MO) exclusion method (Ausubel *et al.*, Short Protocols in Molecular Biology, John Wiley & Sons, Inc., 2nd ed., New York, 1992, p.11-24), and a paired t-test analysis is performed for statistical evaluation of the data.

Example 4: CAT Assay

To measure the expression of the human elastin promoter/CAT reporter gene construct in the skin of transgenic hairless mice and in fibroblast cultures established from these animals, CAT activity is determined. For extraction of the CAT from skin, the specimens are homogenized in 0.25 Tris-HCl, pH 7.5, using a tissue homogenizer (Brinkmann Instruments, Inc., Westbury, NY). The homogenates are centrifuged at 10,000 X g for 15 minutes at 4°C and the protein concentration in the supernatant determined by a commercial protein assay kit (Bio-Rad Laboratories, Richmond, CA). Aliquots of the supernatant containing 100 μg of protein are used for assay of CAT activity by incubation with [^{14}C] chloramphenicol in accordance with well-known procedures. The acetylated and non-acetylated forms of radioactive chloramphenicol are separated by thin-layer chromatography and CAT activity is determined by the radioactivity in the acetylated forms as a percent of the total radioactivity in each sample.

Example 5: UV Sources

For administration of UVB radiation, a closely spaced array of seven Westinghouse FS-40 sunlamps is used which delivers uniform irradiation at a distance of 35 cm. Irradiating with UVA is performed using seven Sylvania FR40T12 PUVA lamps in the above mentioned array, filtered through window glass of 2 mm thickness to remove wavelengths below 320 nm. The energy output at 35 cm is measured with a Solar Light model 3D UVA and UVB detector (Solar Light Company, Philadelphia, PA). The output of FX-40 sunlamps is 23.4 units/hour of UVB at 38 cm, where each unit is equivalent to 21 mJ/cm^2 of erythema effective energy. The output for FR40T12 PUVA lamps filtered through window glass is 2.02 mW/cm^2, with no detectable UVB radiation.

A Multiport Solar Simulator (Solar Light Company, Philadelphia, PA) containing a xenon arc lamp filtered through a Schott WG 320 filter (Schott Glaswerke, Mainz, Germany) is used to administer solar simulating radiation (SSR). The output of the solar simulator is measured by means of a 3D UV meter (Solar Light Company) and displayed as human minimal erythema doses (MEDs). The emission spectrum of the lamp closely simulates solar radiation reaching the earth's surface.

Example 6: Irradiation

Mice are placed under the center of the light array and restrained with adhesive tape, exposing their dorsal surfaces to the ultraviolet radiation at a distance of 35 cm from the fluorescent tubes. For the Multiport Solar Simulator, light guides from the solar simulator are placed in light contact with the dorsal surface of the mice, which are restrained to prevent movement while SSR is administered. Untreated control mice are restrained in a similar manner.

Fibroblast cultures as described above are exposed for 5, 10, 20, 40 and 80 seconds of UVB corresponding to doses of 0.7, 1.4, 2.7, 5.5 and 10.9 mJ/cm^2, respectively. Cultures are exposed to UVA for 2.3, 4.6, 9.2 and 18.4 minutes corresponding to doses of 0.3, 0.6, 1.1 and 2.2 J/cm^2. To prevent light absorption by tissue culture medium, just prior to irradiation, tissue culture medium is removed from cells and replaced with a thin layer of phosphate buffered saline (PBS) sufficient to cover the cells. Control unirradiated cells are also placed in PBS. Medium is replaced in all dishes immediately after the last light dose is administered. Only fibroblasts from mice in the same litters are used for any given experiment and utilized in the first few passages. Two dishes of cells are used for each time point.